MODIFICATION OF X-RAY INJURY TO HYDRA LITTORALIS [1] BY POST-IRRADIATION TREATMENT WITH MAGNESIUM SULFATE AND GLUTATHIONE [2]

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Very few studies have been reported on the damaging effects of ionizing radiations on *Hydra*. Zawarsin (1929), Strelin (1929) and Evlakhova (1946), however, studied the effect of sublethal doses of x-rays and found that inhibition of budding and regeneration varied with the dose of radiation used. Daniel and Park (1951, 1953) reported a toxic effect of x-ray-treated media on *Hydra* tentacles and (1954) direct x-ray damage leading to death in 24 hours.

A number of investigators (Barron *et al.*, 1949; Bellack and Krebs, 1951; Chapman and Cronkite, 1950; Chapman *et al.*, 1950; Patt *et al.*, 1950; Bacq, 1951; Cronkite *et al.*, 1951) demonstrated that glutathione modifies some of the biological effects of ionizing radiations. In general, protection resulted only if the glutathione was given before irradiation. Similarly, in most cases cysteine has to be present at the time of irradiation in order to exert a protective effect (see Patt, 1953). Barron and co-workers, however, found that when glutathione was added to aqueous solutions of succinoxidase after irradiation, the enzyme was partially reactivated. Patt *et al.* (1952) reported protection to mammalian thymocytes when cysteine was added immediately after irradiation.

Daniel and Park (1954) showed that when hydras given 25,000 r were placed immediately in a dilute solution of salts containing either $MgSO_4$ or $MgCl_2$, about twice as many survived 24 hours as were living in the same salt solution without Mg^{++}. In view of this result, and of the few cases reporting modification of x-ray damage by post-irradiation treatment with sulfhydryl compounds, the present studies were made on the effects of continuous post-irradiation treatment with $MgSO_4$ plus glutathione on survival and on budding of hydras.

Materials and Methods

The hydras used in the present studies were from a clone culture grown in the laboratory at a room temperature of 25° ± 1.5° C. The cultures were kept in a standard salt solution of 1.7×10^{-3} *M* NaCl, 5.4×10^{-5} *M* KCl and 3.3×10^{-4} *M* $CaCl_2$ in double-distilled water (the second distillation being from glass). This solution contained the same salts and in approximately the same concentration as

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[2] These data are from a thesis submitted to the Graduate Council of the George Washington University by Helen D. Park in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

that used by Daniel and Park (1954), and will hereafter be referred to as "standard saline." The hydras were fed newly hatched brine shrimp daily and were washed and changed to fresh standard saline one hour after each feeding. They were transferred to clean dishes once a week. Under these conditions the hydras reproduced asexually by budding.

For all experiments, hydras of equal size, without buds, were selected from the stock cultures before the daily feeding and washed in standard saline before treatment. The hydras were irradiated in a Pyrex glass dish containing 50 ml. of standard saline which gave a solution depth of 17 mm. The hydras immediately were washed with standard saline, then within 10 minutes were placed in the solutions to be studied. Except while the hydras were under observation the dishes were kept in moist chambers.

Irradiation factors were 50 kv constant potential, 50 ma beryllium window tube; 2700 r per minute. Dose determinations were made by the method of Andrews and Shore (1950). An aluminum plate 0.020 inch thick served as an x-ray filter and as a dish cover. Water cooling of the irradiation dish kept the temperature of the contents within 2° C. of the temperature of the laboratory.

Results

Survival experiments

In order to compare the effect of glutathione with that of $MgSO_4$, equal numbers of hydras exposed to 25,000 r were placed in (1) standard saline; and (2) 5.0×10^{-4} *M* $MgSO_4$, (3) 1.0×10^{-5} *M* glutathione, and (4) 5.0×10^{-4} *M* $MgSO_4$ plus 1.0×10^{-5} *M* glutathione, each in standard saline. Non-irradiated controls were also treated with the four solutions. The $MgSO_4$ concentration was within the range previously found by Daniel and Park to protect hydras exposed to 25,000 r. Within this range the protective effect of $MgSO_4$ was not a function of the ionic strength of the solutions. The concentration of glutathione had previously been shown to modify a toxic effect of irradiated water on hydra tentacles. The animals were left in their respective solutions 24 hours, at which time the survivors were counted. Five complete experiments, each consisting of 10 animals per group, were carried out in a period of 30 days.

Table I

Effect of post-irradiation exposure to $MgSO_4$ and glutathione on survival of hydras after 25,000 r. Fifty hydras in each treatment group

Treatment	Number alive after 24 hours	
	Irradiated	Non-irradiated
Standard saline	12±2	50
5.0×10^{-4} *M* $MgSO_4$ in standard saline	30±4	50
1.0×10^{-5} *M* glutathione in standard saline	18±4	50
5.0×10^{-4} *M* $MgSO_4$ + 1.0×10^{-5} *M* glutathione in standard saline	21±4	50

Standard error estimated from variation among 5 experiments.

As shown in Table I none of the non-irradiated hydras died. All of the irradiated groups showed by chi square test significantly [3] fewer survivors than their controls. The only statistically significant differences among the irradiated groups were between the hydras in $MgSO_4$ and those in saline, and between those in $MgSO_4$ and those in glutathione. There is not sufficient statistical evidence to assert definitely that either glutathione or the combined treatment had a protective effect against the radiation. The present results confirm the conclusion of Daniel and Park that $MgSO_4$ had a specific protective effect against the radiation.

Budding experiments

Hydras reproduce asexually by the formation of buds which constrict from the parent as adult hydras. The process involves increase in the mass of protoplasm, cell division and differentiation. In the stock cultures maintained in this laboratory, the development of a bud, from the time it is first recognizable until its separation from the parent, takes from two to four days.

The effects of continuous post-irradiation exposure to $MgSO_4$ and glutathione on budding were studied using 4500 r, a dose the author had previously found to be approximately one-third that necessary to inhibit bud production completely for 10 days. Forty irradiated and 40 non-irradiated hydras in groups of 10 were put in the standard saline, $MgSO_4$ saline, glutathione saline and $MgSO_4$ + glutathione saline solutions previously described. Beginning on the first day after irradiation, the hydras were fed daily. All descendents derived from the original 10 hydras in each group were kept with the parents. Each day for 11 days adults and attached buds were counted as separate individuals and all were transferred to fresh solutions in clean dishes. Five experiments, each including all of the treatment groups, were carried out at intervals over a period of two months.

For all analyses [4] of the data, statistical significance or the lack thereof was determined by comparing an average effect over the five replicate experiments with the variation of this effect among the five experiments.

Figure 1 shows, from days zero through eleven, the average number of adults plus buds present per experiment in each treatment group. Among the non-irradiated hydras, those in $MgSO_4$ and $MgSO_4$ + glutathione produced significantly greater numbers of descendents by the end of 11 days than those in standard saline or glutathione. Since neither of the other two intergroup differences among non-irradiated hydras was significant, it seems probable that during the combined exposure it was $MgSO_4$ which caused the increase in budding.

Comparison of the groups in saline alone shows that 4500 r depressed significantly the budding rate. The data for the irradiated hydras in standard saline suggest that the normal budding rate was regained by day nine, but since a parabola does not fit the points better than a straight line, the break in the curve may be fortuitous.

The budding rate of the irradiated hydras in $MgSO_4$ was not significantly greater than that of their irradiated controls. On the other hand, the irradiated hydras in glutathione produced buds at a significantly greater rate than the irradiated

[3] The .05 level of probability was used throughout the present work.

[4] The author wishes to thank Mr. Jerome Cornfield of the National Institutes of Health for his help in analyzing the data statistically.

controls. The irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated controls or the irradiated hydras in $MgSO_4$ or the irradiated hydras in glutathione, and at the same rate as the non-irradiated hydras in standard saline. It can be concluded, therefore, that under the conditions of these experiments, continuous post-irradiation exposure to $MgSO_4$ + glutathione restored the budding rate of irradiated hydras to that of non-irradiated hydras in standard saline.

In addition to showing the budding rates of all the groups, Figure 1 shows, on the

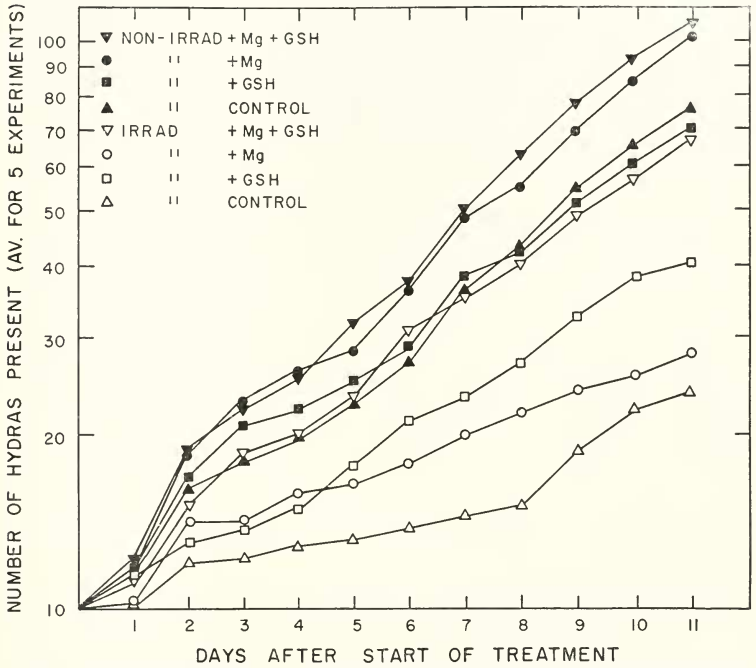

FIGURE 1. Effect of post-irradiation treatment with $MgSO_4$ and glutathione on budding of hydras after 4500 r. Treatment solutions were made in standard saline.

average, the time at which all 10 of the hydras in each group initiated their first buds (*i.e.*, when 20 adults and buds were present in each group). The data for the individual experiments show that all 50 of the non-irradiated hydras in standard saline initiated their first buds by day six. The total number of irradiated hydras in each treatment group that had initiated their first buds by the time all first buds appeared in the non-irradiated standard saline group was: standard saline 17, $MgSO_4$ 39, glutathione 35, and $MgSO_4$ + glutathione 47. It can be concluded that one of the effects of the radiation was to delay the time of appearance of first buds.

Magnesium sulfate, glutathione, and $MgSO_4$ + glutathione reduced the severity of the radiation effect, but only the combined treatment shortened the time of first bud initiation to that of the non-irradiated, saline controls.

Since the irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated hydras in $MgSO_4$ alone or glutathione alone, the effect of concentration of the two agents on budding after irradiation was studied in order to ascertain whether the greater effect produced by the two agents together is valid when related to optimal effects of each when used separately. Accordingly, hydras were irradiated with 4500 r and placed in groups of five in solutions in which both $MgSO_4$ and glutathione concentrations were varied from ½ to 16 times those used in the preceding experiments. The hydras were counted on the eleventh day after irradiation.

Table II shows the mean number of hydras present on the eleventh day in each treatment group. The results presented in column 1 show that up to a concentration of 8.0×10^{-3} mole per liter, $MgSO_4$ did not modify the inhibiting effect of 4500 r of x-rays. The results shown in line 1 of the table indicate that glutathione in

TABLE II

Effect of concentration of $MgSO_4$ and glutathione on budding of hydras after 4500 r. Mean number of individuals present on 11th day post-irradiation per 5 hydras treated

Moles per liter of $MgSO_4$	Moles per liter of glutathione						
	0.0	5.0×10^{-6}	1.0×10^{-5}	2.0×10^{-5}	4.0×10^{-5}	8.0×10^{-5}	1.6×10^{-4}
0.0	5.0(5)	6.5(4)	9.0(4)	6.8(4)	10.0(4)	5.0(3)	0.3(3)
2.5×10^{-4}	5.5(4)	13.0(3)	11.3(3)	12.3(3)	11.0(3)	6.0(3)	2.0(3)
5.0×10^{-4}	5.0(5)	9.3(3)	13.0(2)	17.3(3)	19.0(3)	8.3(3)	4.0(3)
1.0×10^{-3}	5.2(4)	9.3(3)	12.0(3)	10.3(3)	13.0(3)		1.7(3)
2.0×10^{-3}	5.4(5)		12.0(3)		22.0(4)	5.0(3)	
4.0×10^{-3}	5.7(3)		10.3(3)		14.0(1)	10.0(3)	1.0(1)
8.0×10^{-3}	5.0(3)		6.0(2)			6.0(2)	4.0(2)

Figures in parentheses = number of groups of hydras treated. The mean number of individuals present on the 11th day in 5 groups of 5 non-irradiated hydras in standard saline was 12.0. No concentration tests were run on non-irradiated hydras.

concentrations between 5.0×10^{-6} and 4.0×10^{-5} mole per liter reduced the inhibitory effect of the radiation on bud production; 1.6×10^{-4} *M* glutathione was highly toxic. Radiation was probably not a factor in this toxicity as five non-irradiated hydras placed in this solution were dead five days later. The data show that the optimal concentrations of the two agents when supplied together were in the range of 2.0×10^{-5} to 4.0×10^{-5} *M* glutathione and 5.0×10^{-4} to 2.0×10^{-3} *M* $MgSO_4$. In addition they show that combined treatment within these ranges resulted in greater bud production than at optimal concentrations of either agent used separately. Furthermore, the amount of budding that took place during exposure to optimal concentrations of both agents together was as great as that of the non-irradiated hydras in standard saline.

DISCUSSION

At first glance the effects of $MgSO_4$, glutathione and combined treatment, after the two radiation exposures employed, appear to be anomalous. Since the two sets

of results are expressed in different units—number surviving out of total number treated, and rate of increase in numbers of hydras present—they cannot be compared statistically. Taking the apparent discrepancies at face value, however, it seems reasonable that a particular agent might be more effective against a mild cellular damage which would partially inhibit budding than against a more drastic injury leading to death in 24 hours, or that another agent might be more effective in keeping an animal alive for 24 hours than in maintaining it in a reproductive state for a period of 10 days.

The mechanism of the stimulating action of $MgSO_4$ on budding of non-irradiated hydras is not known. However, this effect is perhaps not surprising in view of the fact that $MgSO_4$ has been shown to affect growth in many organisms as widely separated phylogenetically as bacteria (Webb, 1953), protozoa (Mast and Pace, 1939), and mammals (Kruse *et al.,* 1932). Since the addition of increasing amounts of $MgSO_4$ did not increase the amount of budding of irradiated hydras, we may conclude that lack of $MgSO_4$ was not the factor which limited budding after irradiation.

Mechanisms of radiation protection have been considered in reviews by Ord and Stocken (1953) and by Patt (1953). One of the theories of protection by sulfhydryl compounds is that there is a competition by —SH groups for free radicals formed from water in an irradiated solution. Since, in the study reported here, the hydras were not irradiated in the presence of glutathione, and were washed immediately after irradiation and at least ten minutes elapsed between the end of irradiation and beginning treatment with glutathione, the effect on budding would seem to have been due to some mechanism other than a competition of —SH groups for free radicals within or at the surface of the hydra cells.

It is not known whether hydras need an external source of glutathione for budding. If they do, it is possible that the reason glutathione did not stimulate the budding of the non-irradiated hydras was because they were already getting a sufficient amount for budding in their normal intake of food. If hydras do not need an external source of glutathione for budding, stimulation would not occur on the addition of glutathione to the medium.

It was not practicable to determine the amount of food eaten by any of the hydras. However, if the irradiated hydras ate less food than the controls, the rate of budding would be reduced from that of the controls. The effect of glutathione in increasing the budding rate of irradiated hydras might thus have been due to the fact that this agent stimulates mouth opening (Loomis, 1955) which in turn might permit the hydras to consume more food. A second possibility is that the requirement of irradiated hydras for glutathione or some part of the molecule may be greater than that of non-irradiated hydras, *e.g.,* because of the reconstitution of injured regions. Thus the requirement might not be met even with normal food intake, causing a decreased budding rate; addition of glutathione to the medium might satisfy the greater requirement and increase the budding rate over that of the irradiated controls.

The fact that $MgSO_4$ stimulated budding of non-irradiated hydras that were presumably getting an adequate amount of glutathione through their normal intake of food, and the fact that none of the concentrations of $MgSO_4$ used after irradiation had a significantly stimulating effect unless added glutathione was present,

suggest the possibility that in all hydras, stimulation of budding by $MgSO_4$ depends on the presence of an adequate level of glutathione or sulfhydryl in the hydra tissues.

Summary

1. Hydras were left for 24 hours in solutions of $MgSO_4$, glutathione and $MgSO_4$ + glutathione after exposure to 25,000 r x-rays. Only the hydras in $MgSO_4$ alone were significantly protected against the effects of the radiation.

2. Hydras were exposed continuously to $MgSO_4$, glutathione, and $MgSO_4$ + glutathione solutions after 4500 r. The rates of budding in each solution were determined. It was found that:

(a) Forty-five hundred r inhibited the budding of hydras significantly.
(b) Magnesium sulfate and $MgSO_4$ + glutathione stimulated budding of non-irradiated hydras while glutathione alone did not.
(c) Magnesium sulfate alone did not significantly modify the inhibitory effect of the radiation on budding.
(d) Glutathione alone partially reversed the inhibitory effects of the x-rays.
(e) Within a fairly wide range of concentrations of $MgSO_4$ and glutathione, the two agents together restored the budding rate of irradiated hydras to that of the non-irradiated animals in standard saline.

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